Myc and Mad bHLHZ domains possess identical DNA-binding specificities but only partially overlapping functions *in vivo*

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The opposing transcriptional activities of the basic-helix–loop–helix-leucine zipper proteins Myc and Mad, taken together with information related to their expression patterns and biological effects, have led to a model of the Myc/Max/Mad network in which Myc and Mad proteins function as antagonists. This antagonism is presumed to operate at the level of genes targeted by these complexes, where Myc:Max activates and Mad:Max represses expression of the same set of genes. However, a detailed analysis of the DNA-binding preferences for Mad proteins has not been performed. Furthermore, the model does not address the findings that Myc:Max indirectly represses transcription of several regulatory genes. To examine these issues relating to DNA-binding specificity and biological responses, we have determined the DNA-binding preferences of Mad1 by using selection and amplification of randomized oligonucleotides and demonstrated that its intrinsic specificity is identical with that of c-Myc. We have also used a chimeric Myc protein, containing a substitution of the entire Mad basic-helix–loop– helix-leucine zipper motif, and shown that it can reproduce the growth-promoting activities of Myc, but not its apoptotic function. Our results suggest that Myc and Mad, although possessing identical *in vitro* DNA-binding specificities, do not have an identical set of target genes *in vivo*, and that apoptosis is one biological outcome in which the transcriptional effects of Myc are not directly antagonized by those of Mad.

The Max transcription factor network (comprising Max, the Myc and Mad family proteins, and Mnt and Mga) is profoundly involved in the regulation of cell growth and proliferation (for recent reviews, see refs. 1–3). All members of the Max network contain a conserved basic-helix–loop–helix-leucine zipper (bHLHZ) motif that facilitates association with Max and the subsequent binding of Max heterodimers to DNA.

Myc proteins also contain conserved N-terminal regions, termed "Myc box I" and "Myc box II," which contribute to Myc transcriptional activities by recruitment of a coactivator complex containing a histone acetyltransferase (ref. 4; for recent reviews, see refs. 1 and 5). Myc:Max complexes can activate transcription when bound to DNA at the consensus sequence CACGTG (4, 6–8). In addition to CACGTG binding, Myc:Max complexes can bind to the noncanonical sites CATGTG, CA**T**G**C**G, CACG**C**G, CACG**A**G, and C**AA**CGTG (noncanonical nitrogenous bases indicated in bold) (9). Myc proteins are also thought to play a role in the transcriptional repression of specific genes (10, 11), and recent experiments indicate that Myc repression occurs through the inactivation of the initiator-binding protein Miz-1 (8, 12, 13). In contrast to Myc, Mad proteins are dedicated repressors. All Mad family proteins possess a short N-terminal domain that is required for transcription repression through association with the highly conserved corepressors known as mSin3A and mSin3B (15–19).

Myc family proteins promote cell proliferation, whereas the proteins comprising the Mad family (Mad1, Mxi1, Mad3, and Mad4) seem to function to limit proliferation. Although targets of Myc activation and repression have been identified which help explain Myc-associated activities (including cell cycle progression, cell growth, and apoptosis) it remains unclear which genes are critical for Myc's biological effects (for review, see ref. 1). Few reports have been published regarding Mad target genes. Recently, DePinho and coworkers characterized the target genes of a Myc fusion protein in which the basic region was replaced with that of the Mad family member Mxi1 (20). However, this domain replacement contained only the basic region of Mxi1 and not the entire bHLHZ. Because regions other than the basic region seem to contribute to the binding specificity of bHLH proteins (28, 29), substitution of the basic region alone may not accurately reflect the target gene specificity of Mxi1.

A central question is whether Myc and Mad have opposing biological functions as the result of contrasting action on the same genes. To determine the degree to which the sets of Myc and Mad target genes overlap, we have used three approaches. We have assessed the intrinsic DNA-binding preferences of Mad, investigated whether DNA modification affects Myc and Mad in a similar manner, and determined whether a domain replacement involving the intact bHLHZ of Mad can reproduce the biological effects of Myc.

Materials and Methods

Synthesis of Nucleotides and Primers. A random pool of oligonucleotides was synthesized as a single 71-bp oligonucleotide of sequence 5′-AGAATAGGATCCGAATTCTTCTAGAG $(N)_{20}$AGTCGACCCCTAGAAGCTTATTCGG. A 1:1:1:1 mixture was prepared to synthesize the central 20 nucleotides, and the random pool was made double-stranded.

Selection Conditions. For the first round of the selection, 150 ng of double-stranded randomized pool (sufficient for representation of each possible 20-mer twice) was added to 3 μl of TNT rabbit reticulocyte lysate (Promega) and brought to 20 μl with 5 mM DTT/20 mM Hepes (pH 7.7)/10 mM $MgCl_2$/100 mM KCl/0.5 mg/ml BSA/0.15 μg/μl poly(dI-dC)·poly(dI-dC) (Pharmacia)/0.2% Nonidet P-40/6% glycerol/0.05% xylene cyanol, then incubated for 30 min at 25°C. Flag-agarose beads (Sigma) (5 μl per reaction) were prewashed three times, resuspended in 150 μl of the salt/DTT/BSA, combined with each protein/DNA mixture, and allowed to incubate at 4°C for 1 h. Beads were then washed six times on ice, resuspended in 50 μl of distilled/deionized H_2O, and boiled for 5 min. pCITE-F-Myc(MadbHZ) and pVZ-Max were used for *in vitro* transcription and translation (TNT rabbit reticulocyte lysate).

PCR. PCR was performed in multiples of a standard 10-μl reaction: 1 μl of the 50-μl eluate, 0.5 pmol/μl each primer, 200 μM dNTP mix, 0.15 μl of *Pfu* DNA polymerase (Stratagene), 1× Pfu buffer. Ten-microliter aliquots of each reaction

Abbreviation: bHLHZ, basic-helix–loop–helix-leucine zipper.

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were removed at 15, 20, 25, or 30 cycles and resolved on a 3% agarose gel. The number of cycles at which a band was visible on the gel was used to generate PCR products for use in the subsequent rounds of the selection, in which 10 μl of the PCR product was combined with the TNT proteins. After six rounds, PCR products were cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced by using the M13 reverse primer and automated fluorescent sequencing (ABI PRISM, Perkin–Elmer).

Sequences were analyzed (*i*) manually, (*ii*) by using the CLUSTAL X (1.64b) program, and (*iii*) with the algorithms devised by Gary Stormo at the University of Colorado at Boulder (8).

Cloning. Myc and Myc(MadbHZ) were each cloned into the pME18F vector, which encodes the Flag octapeptide at the N terminus. The junction between the Myc protein and the Mad bHLHZ had the sequence PRSSDTEE-SSSRST, and the Mad bHLHZ was made to terminate with the sequence LGIERIADP.

Transient Transfection Assays. NIH 3T3 cells were plated in 24-well plates at 20,000 cells per well in DMEM + 10% bovine calf serum and transfected by using FuGENE-6 (Roche Molecular Biochemicals) 24 h later with the indicated quantity of protein expression vectors, 1 μg of pGL2-M4-luciferase, and 0.1 μg of pRSV-β-galactosidase. After 48 h, cells were washed and then lysed in 75 μl of luciferase lysis buffer [0.65% Nonidet P-40/10 mM Tris·HCl (pH 8.0)/1 mM EDTA/150 mM NaCl]. For luciferase assays, 20 μl of lysate was transferred to a luminometer cuvette and mixed with 300 μl of luciferase reaction buffer [25 mM glycylglycine (pH 8.0)/5 mM ATP/15 mM $MgSO_4$]. Relative luminescence units were measured for 10 s in the luminometer after automatic injection of 100 μl of 3 mM luciferin diluted in 25 mM glycylglycine, pH 8.0. For β-galactosidase assays, 20 μl of lysate was measured with the standard Z buffer MUG protocol (46). Experiments were performed in triplicate.

Cells and Transfections. To create stable cell lines, cells were transfected by using the FuGENE-6 kit at 1:10 of protein expression plasmid/drug-resistance plasmid (pBabe-puro). After 48 h, cells were maintained in a concentration of puromycin at which untransfected cells were not viable. For determination of protein expression equal numbers of cells were grown logarithmically and labeled with [^{35}S]methionine (NEN) for 1 h. Proteins were immunoprecipitated as described (15).

DNA and RNA Content. To calculate DNA content, cells were fixed with ethanol, stained with propidium iodide at 50 μg/ml, and measured with a Becton Dickinson FACScan analyzer, and cell distribution was determined by using MULTICYCLE. To calculate RNA content, equal numbers of cells were washed once with PBS and resuspended in PBS/2% bovine calf serum. Of the cell suspension 0.2 ml was combined with 0.4 ml of solution A (0.1% Triton X-100/80 mM HCl/150 mM NaCl) and, after 10 min, 1.2 ml of solution B (6 μg/ml acridine orange/1 mM EDTA/150 mM NaCl/37 mM sodium citrate/126 mM Na_2HPO_4, pH 6.0). During analysis, cells were gated by using forward light scatter and side scatter to remove debris, and G_1 cells were isolated by using FL-1 (DNA) vs. FL-3 (RNA).

Results and Discussion

Identification of Preferred Mad1 DNA-Binding Sites. The DNA-binding specificity of Myc:Max heterodimers was originally defined from their preferential binding to E-box sequences among populations of completely randomized oligonucleotides (9, 21, 22). Although Mad:Max binds to E-box sequences *in vitro* (15), the intrinsic binding preferences of Mad:Max dimers have not been reported. It is conceivable that Mad possesses a specificity distinct from that of Myc. Thus, we performed an *in vitro* binding site selection to determine which DNA sequences are preferentially bound by Mad when presented with oligonucleotides containing all possible sequence combinations (22, 23).

Selection was performed with a pool of oligonucleotides containing a central region of 20 randomized nucleotides surrounded by two regions of defined sequence 25 nucleotides in length. Flag epitope-tagged Mad1 (flg-Mad1) was transcribed and translated *in vitro* with untagged Max and mixed with the oligonucleotide pool, and Mad-Max DNA complexes were separated from unbound DNA by anti-Flag immunoprecipitation. To control for nonspecific antibody binding, untagged Max alone was assayed in parallel as a negative control. We monitored the progress of the selection by using PCR of 15, 20, and 25 cycles to verify selective enrichment of flg-Mad1 over the control. PCR products from the earliest PCR cycle that produced a visible band on an agarose gel were used as starting material for the subsequent round. After six rounds of selection, the PCR products were cloned into a plasmid vector and sequenced (see *Methods and Materials*). Fig. 1*A* shows the sequence data from the negative control and from the flag-Mad PCRs. None of the negative control sequences contains an E-box. In contrast, all but two of the flag-Mad selected sequences possess E-boxes, and all but one of these (FMad1) matches the canonical Myc E-box CACGTG. In addition to searching for E-boxes, we analyzed the data to identify other Mad-specific sequences but found none.

In addition to providing information about E-box binding, the data from the selection were used to identify flanking sequence preferences for Mad (Fig. 1*B*). Because the canonical Mad E-box is palindromic, the 5′ and 3′ flanking sequences were combined to provide a consensus half-site for Mad. Taken together with the core hexamer-binding data, the preferred dodecamer-binding site for Mad is predicted to be GACCACGTGGTC, which matches the preferred consensus site for Myc identified by others (24–27). No preferences were noted for any additional base pairs farther from the E-box.

On the basis of their use of a chimeric Myc protein containing a basic region from Mxi1 in transient transfection assays, DePinho and colleagues (20) concluded that Myc and the Mad family protein Mxi1 differ in their preferences for sequences flanking the central E-box. We believe that the discrepancy with our findings is because these authors used only the basic region of Mxi1 linked to the Myc HLHZ domain in their chimeric protein. Studies of the DNA-binding activity of other bHLH proteins have revealed that changes in both helix 1 and the loop region can influence which sequences are bound by the basic region (28, 29). Thus, although the basic domain is the only region that makes direct contact with the nitrogenous bases of the E-box, it is unlikely to function independently of the HLH and zipper domains.

Mad DNA Binding Is Sensitive to CpG Methylation. Although the binding sites for Myc and Mad seemed to be identical, we hypothesized that DNA binding at target genes might be affected by DNA modification. For example, mammalian genomes have undergone cytosine methylation of CpG dinucleotides within regions of inactive chromatin. Indeed, c-Myc and Max and Myc-Max heterodimers have been described to exhibit methylation-sensitive binding to the consensus sequence CA^{m}CGTG (24, 30). To determine whether Mad possesses the ability to distinguish between methylated and unmethylated sequences, we performed electrophoretic gel mobility-shift assays with oligonucleotides chemically synthesized to contain either a methylated or an unmethylated E-box.

The results demonstrated that Myc:Max and Mad:Max heterodimers bound to unmethylated CACGTG, but not to methylated CA^{m}CGTG (Fig. 1*C*).

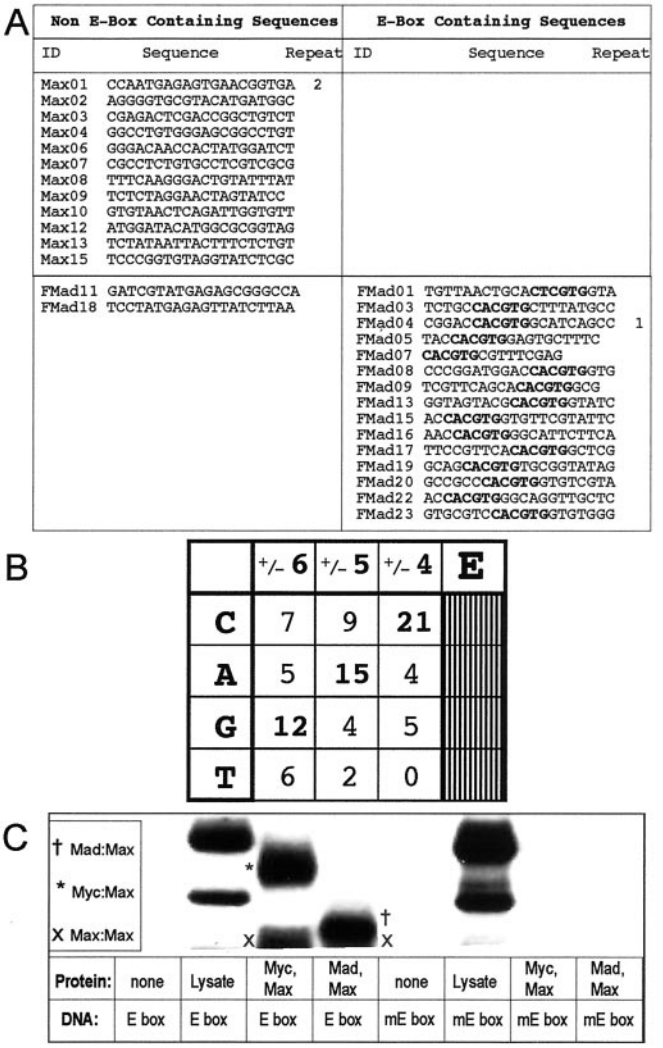

Fig. 1. Preferential binding of Mad1 protein to canonical E-boxes. (*A*) 20-mer sequences independently derived from the SAAB (selected and amplified binding site) selection. The upper portion contains the first 15 sequences from the negative control (untagged Max protein followed by immunoprecipitation with anti-Flag), none of which contain E-boxes. Sequence Max01 was found in two other clones, as denoted to the right of the sequence. The lower portion contains the first 23 sequences from the Flag-tagged Mad selection. Sequences without E-boxes appear on the left, whereas those with E-boxes (in bold) appear on the right. (*B*) Flanking sequences from Mad-selected E-boxes. Nucleotides represented in the +/−4, 5, 6 flanking positions, relative to the central G (+1) and C (−1), are shown, with the most frequently appearing nucleotide in bold. Because the E-boxes (except FMad01) are palindromic, only half-sites are shown. (*C*) Gel-shift assays using labeled nonmethylated E-box, or CpG-methylated E-box (mE box) oligonucleotides. Labeled probes were incubated with the indicated *in vitro* translated proteins.

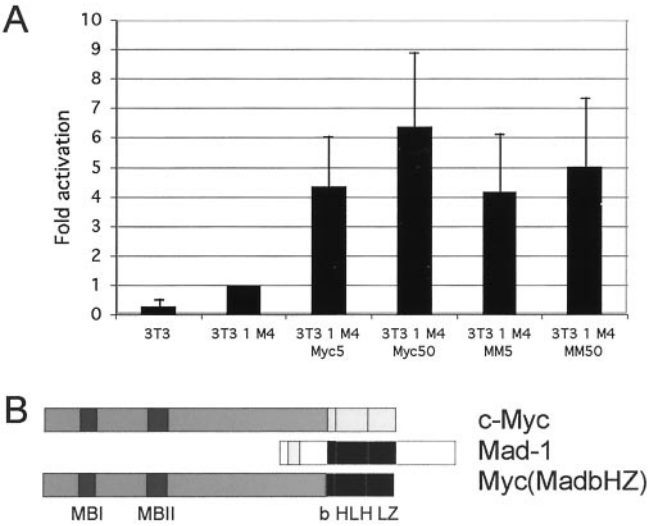

Fig. 2. Myc and the Myc(MadbHZ) fusion protein (MM). (*A*) Transcription assays using a synthetic promoter with four E-boxes controlling expression of the luciferase gene. Assays were performed after transient transfection of NIH 3T3 cells with 1 μg of the M4 reporter (3T3 1 M4), 5 or 50 ng of protein expression vector (Myc5 and Myc50 for Myc; MM5 and MM50 for the Myc(MadbHZ) chimeric protein), and 0.1 μg of RSV-β-galactosidase vector. Luciferase relative luminescence units were divided by β-galactosidase units to correct for variation in transfection efficiency. (*B*) Schematic diagram of the c-Myc protein (N-terminal Myc box I and Myc box II in dark gray and the bHLHZ in light gray), the Mad1 protein [N-terminal Sin3 interaction domain (SID) in light gray, and the bHLHZ in black], and the Myc(MadbHZ) chimera, comprising the entire Myc protein with the exception of the bHLHZ, which has been replaced with the analogous motif from Mad1.

A Myc Protein Containing the Mad1 bHLHZ Domain Activates Transcription of an E-Box Reporter Gene. The experiments described above suggest that the specificities for DNA recognition by Myc and Mad proteins are identical in our *in vitro* assays. To address the question of whether the bHLHZ region of Mad directs biological functions that are different from Myc, we generated a protein in which the C-terminal bHLHZ region of c-Myc is replaced by the cognate region derived from Mad1 (Fig. 2*B*). We used this fusion protein, Myc(MadbHZ), to determine whether its biological activities could recapitulate those of wild-type Myc.

In transient transcription assays, Myc is capable of transcriptional activation of a synthetic reporter containing promoter-proximal E-box-binding sites (7, 31, 32). We therefore compared the transcriptional activity of wild-type c-Myc with that of the Myc(MadbHZ) fusion protein. Fig. 2*A* shows the transcriptional activities of Myc and Myc(MadbHZ), relative to the reporter alone. Addition of 5 ng of Myc or Myc(MadbHZ) transactivates the reporter roughly 4-fold with only a slight increase upon transfection of higher concentrations of expression vector. The effects observed here are consistent with the weak transcriptional activity of Myc observed by us and others (7, 31, 32). We conclude that Myc(MadbHZ) is capable of activating transcription in an E-box-dependent manner to the same extent as Myc.

The Chimeric Myc(MadbHZ) Protein Stimulates Growth and Proliferation of *myc*-Null Cells. To assess the functions of the fusion protein further, we used a Rat-1 cell line in which both c-*myc* alleles have been deleted by targeted homologous recombination (33, 34). The c-$myc^{-/-}$ HO15.19 cells contain no detectable *myc* family gene products (34). These Myc-null cells display a distinct flattened morphology, relative the TGR-1 parental Rat-1 cells, and divide at a slower rate (doubling time approximately 50 h compared with 20 h for TGR-1 cells). Reintroduction of c-*myc* significantly decreases the doubling time to TGR-1 levels or less (34).

HO15.19 $myc^{-/-}$ cells were cotransfected with a drug-resistance plasmid and either of the Flag-epitope-tagged constructs F-Myc or F-Myc(MadbHZ). Resistant cells were selected, pooled, labeled with [^{35}S]methionine, and subjected to immunoprecipitation with anti-flag. Fig. 3*A* demonstrates that these pools of cells express roughly equivalent levels of the two tagged proteins. To assess the ability of Myc(MadbHZ) to rescue the proliferation defect of the c-*myc*-null cells, proliferation rates and cell cycle distributions were determined. Equal numbers of logarithmically growing parental TGR-1, $myc^{-/-}$, and $myc^{-/-}$ cells expressing F-Myc and F-Myc(MadbHZ) were cultured and

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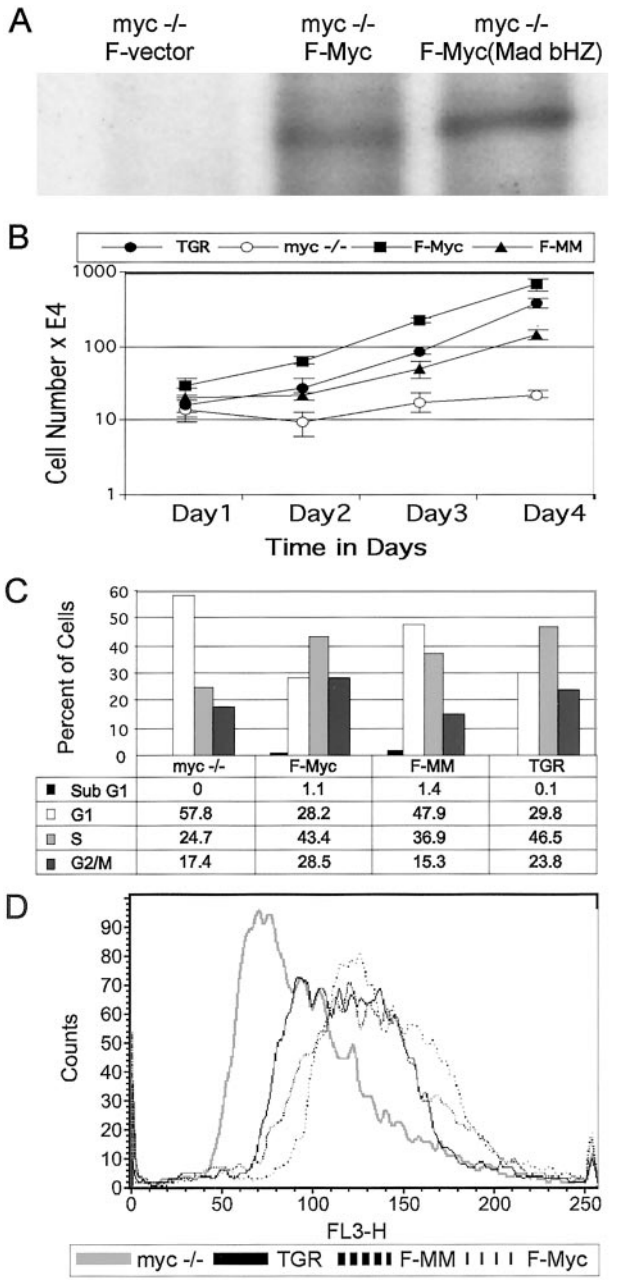

Fig. 3. Characterization of activity of Myc(MadbHZ) and Myc in stably transfected $myc^{-/-}$ cells. (*A*) Equivalent expression of F-Myc and F-Myc(MadbHZ) (MM) proteins. Pools of stably transfected cells were labeled with [^{35}S]methionine, and proteins were immunoprecipitated with anti-Flag M2 antibody then resolved on an SDS/12.5% polyacrylamide gel. The figure shows the pertinent segment of the autoradiograph. (*B*) Proliferation of transfected Rat-1 cell. Equal numbers cells were plated on day 0 and then trypsinized and counted at days 1, 2, 3, and 4. (*C*) DNA content profiles of logarithmically growing cells. The graph contains the cell cycle distribution data from MULTICYCLE analysis of flow cytometry data from cells stained with propidium iodide. (*D*) Both Myc and Myc(MadbHZ) restore the levels of RNA in $myc^{-/-}$ cells. The graph shows an overlaid set of histograms representing the G_1 RNA content of cells stained with both propidium iodide and acridine orange.

counted on successive days. Fig. 3*B* shows the data plotted on a semilogarithmic scale. The reduced division rate of the $myc^{-/-}$ cells is evident when compared with that of the parental TGR cells. The slope for cells expressing Myc(MadbHZ) from day 2 onward is equal to that for Myc, and both of these values are slightly lower than the parental TGR cells.

To measure more precisely the rescue of cellular proliferation during logarithmic growth, DNA content was determined using flow cytometry. The tabulated data showing cell cycle distribution are presented in Fig. 3*C*. Similar to previous reports, the $myc^{-/-}$ cells have a reduced S phase fraction relative to the TGR cells, and overexpression of Myc or Myc(MadbHZ) results in an increased S phase population in the *myc* null cells.

An earlier analysis of the $myc^{-/-}$ Rat-1 cells had noted a decreased rate of growth as determined by measurement of RNA and protein synthetic levels (34). Because regulation of cell growth is thought to constitute a major activity of Myc (35–38), we used acridine orange to examine whether the F-Myc and F-Myc(MadbHZ) proteins would influence RNA accumulation. Fig. 3*D* shows the relative G_1 RNA content distributions within our experimental cell populations. Although the $myc^{-/-}$ cells have a markedly reduced amount of cellular RNA relative to the TGR cells, the introduction of either Myc or Myc(MadbHZ) results in an increase in the average RNA level within the cell population. Treatment with RNase A abolishes the signal, thus confirming that the results are specific for determination of RNA levels (data not shown).

In these experiments the Myc(MadbHZ) protein reproduced many of the effects of Myc in cells. It transactivated E-box-driven artificial promoter, rescued the proliferation defect of *myc* null cells, increased the percentage of cells in S phase, and stimulated cell growth. Because the fusion protein contains the Mad1 bHLHZ, these results are consistent with the notion that Mad binds to the Myc target genes responsible for these biological effects.

The Chimeric Myc(MadbHZ) Protein Does Not Stimulate Apoptosis. In our characterization of the $myc^{-/-}$ Rat-1 cell line, it was noted that under growth-arrest conditions a significant portion of the cells accumulated in G_2/M (23%) in contrast to the G_1 accumulation (91%) observed for arrested TGR cells (34). To determine whether F-Myc and F-Myc(MadbHZ) differed in their ability to affect cell cycle phasing during growth arrest, we analyzed cells arrested by contact inhibition in the presence of normal serum conditions (10% FBS). Cells were plated at about 80% confluence and grown to complete confluence in the course of 1 week, during which time the media were changed every 48 h. Once at confluence, cells were harvested and analyzed for cell cycle distribution. The data in Fig. 4*B* demonstrate that the $myc^{-/-}$, TGR, and F-Myc(MadbHZ) cells have all arrested, as seen by their dramatically reduced S phase fraction. In contrast, 20% of the F-Myc cells are in S phase, indicating that these cells did not completely withdraw from the cell cycle despite their growth to high density.

F-Myc-expressing cells also possess a substantial sub-G_1 population (18.6%), representing cells undergoing apoptosis, not found when F-Myc cells are grown at logarithmic phase (1.1%) (see Fig. 3*C*). For $myc^{-/-}$, TGR, and F-Myc(MadbHZ), the rates of apoptosis are very low. Thus, when overexpressed, F-Myc seemed to override the growth-arrest signals of confluence and to drive cells into S phase. However, this continued division also resulted in significant cell death. Thus, although F-Myc(MadbHZ) seemed to rescue the proliferation defect of Myc-null cells during logarithmic growth and reverse the G_2/M block seen during growth arrest, it does not completely mimic the functions of F-Myc. The discrepancy between Myc and Myc(MadbHZ) in apoptotic function prompted a further examination of apoptotic effects of the two Myc constructs.

Earlier studies have demonstrated that Myc can accelerate apoptosis induced by radiation damage or by withdrawal of cytokines or serum (39–41). We induced apoptosis by growing cells to confluence and then adding medium containing either

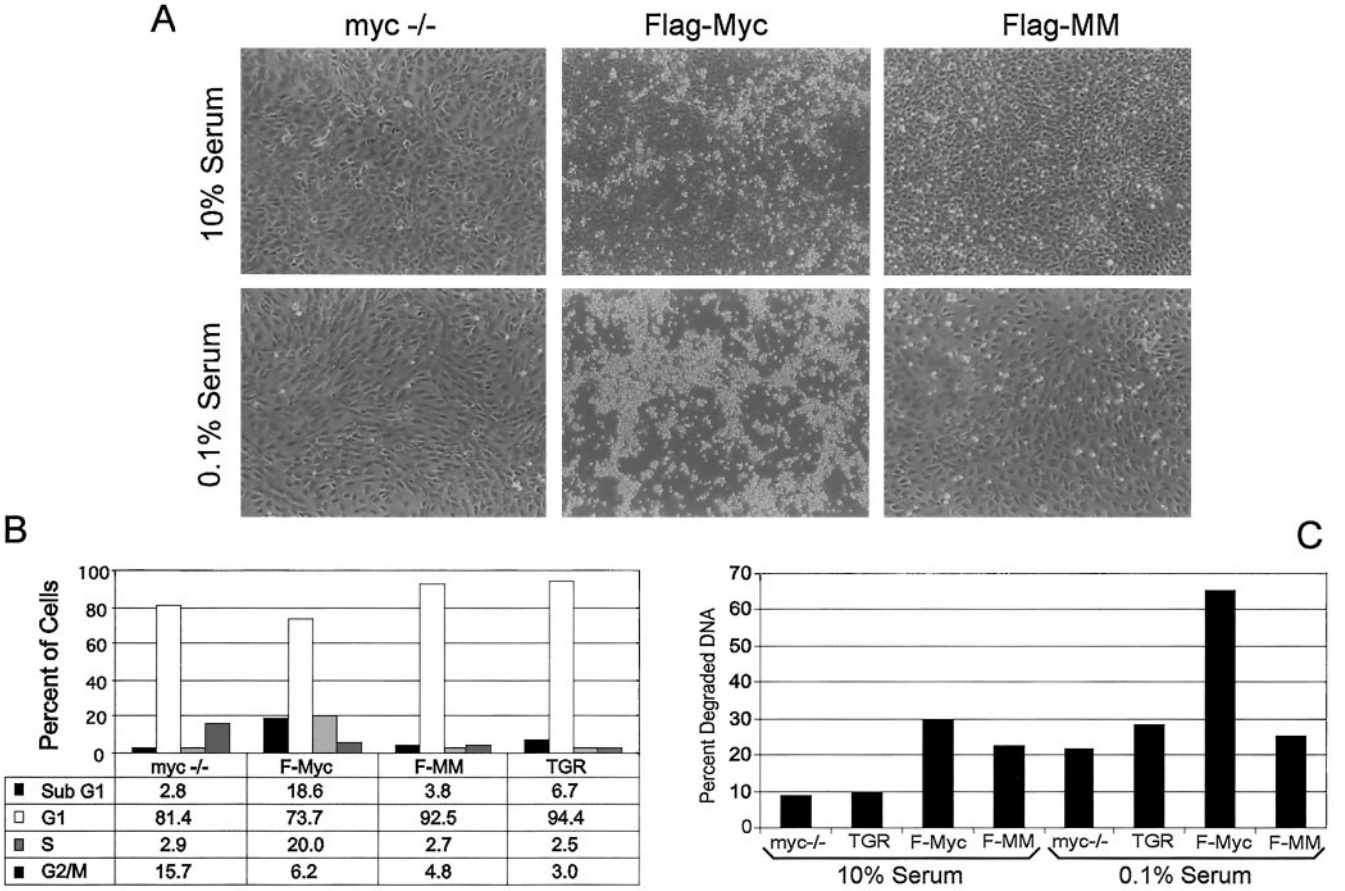

Fig. 4. Myc, but not Myc(MadbHZ), induces apoptosis. (*A*) Photomicrographs of Rat-1 cells following growth-factor withdrawal. Cells were grown to confluence, washed with PBS, and then refed with medium containing 10% or 0.1% serum. (*B*) DNA content profiles of density-arrested cells. The graph contains the cell cycle distribution data from MULTICYCLE analysis of flow cytometry data from cells stained with propidium iodide. Note the high S phase fraction of Myc-rescued cells is associated with a high sub-G_1 fraction. (*C*) Quantitation of DNA degradation shows apoptosis induced by Myc. The amount of intact and degraded DNA was quantitated as described (42) and used to calculate the percent degraded DNA.

10% or 0.1% serum. Cells were analyzed at 24-h intervals. Fig. 4*A* shows photomicrographs taken at day 3. In 10% serum, the $myc^{-/-}$ cells and the F-Myc(MadbHZ) Rat-1 cells show little evidence of rounded refractile cells typical of those undergoing apoptosis. The F-Myc-rescued population displays a larger number of presumptive apoptotic cells. In 0.1% serum, the $myc^{-/-}$ and Myc(MadbHZ) cells are largely unchanged and show few signs of apoptosis. In contrast, the vast majority of cells expressing F-Myc are detached from the plate, highly refractile, and extremely small, all signs characteristic of apoptotic cells (40).

Cells undergoing apoptosis are known to degrade genomic DNA into multiples of 200 bp, producing a "DNA ladder" visible on agarose gels (42). To confirm that a significant fraction of these cells are actually undergoing apoptosis, we quantitated the degree of genomic DNA degradation as described in *Materials and Methods*. Fig. 4*C* is a plot showing the percent of low molecular weight DNA in the different cell lines. The data demonstrate that a significant amount of genomic DNA (about 65%) is degraded in F-Myc-expressing cells when serum is withdrawn. In contrast, the percent of degradation in the Myc-(MadbHZ) is equivalent to the $myc^{-/-}$ and the TGR source cells. These data confirm the results obtained with Myc and Myc-(MadbHZ) under conditions of high-density growth arrest; overexpression of the Myc(MadbHZ) fusion protein fails to restore the sensitivity to apoptosis associated with overexpression of wild-type Myc.

To exclude the possibility that the apoptotic effects were the result of using a particular $myc^{-/-}$ cell line, we used an independent, low-passage Rat-1 cell line to repeat the experiment. Rat-1 cells were stably transfected with a plasmid encoding drug resistance and either a vector control, Myc, or Myc(MadbHZ). The apoptosis experiment was repeated, and identical results were obtained as with the $myc^{-/-}$ cells (data not shown), which demonstrates that the apoptotic properties of Myc and Myc-(MadbHZ) are not restricted to the $myc^{-/-}$ cell lines but are rather a reflection of the biological properties of these proteins in fibroblasts.

Uncoupling of Proliferation and Apoptosis. The ability of Myc(MadbHZ) to recapitulate the proliferative and growth effects of Myc (Fig. 3) but not its apoptotic effects (Fig. 4) suggests that these functions are separable. Nonetheless, under apoptosis-inducing conditions we note a decreased S phase fraction in the Myc-(MadbHZ) cells, and parental TGR cells, relative to cell-expressing Myc (Fig. 4). Thus, the ability to induce S phase under serum-limiting conditions may be related in an as-yet-unknown manner to Myc's ability to induce cell death. The notion that the proliferative and apoptosis functions of Myc can be uncoupled are consistent with two previous reports in which mutations within the N terminus of c-Myc were observed to have differential effects on Myc-induced proliferation, transformation, and apoptosis (43, 44). Our data are also consistent with a study showing that, although the Mad1 protein can attenuate apoptosis, it does not seem to do so by blocking the ability of Myc to stimulate apoptosis through cytochrome *c* release (45).

Taken together, these data imply that distinct target genes and/or functions are involved in the proliferative and apoptotic responses to Myc. The effects of point mutations in the N-terminal region of c-Myc have been taken as evidence that a specific transcriptional activity, most notably repression, is involved in driving apoptosis (44). Thus, one explanation for an uncoupling of proliferation and apoptosis is that the target genes whose expression is modulated by Myc to accelerate apoptosis may be distinct from those involved in the proliferative response. Because the Myc(MadbHLHZ) protein would be expected to retain the transcriptional activities of c-Myc, the inability of the chimeric protein to induce apoptosis may be related to its inability to recognize and modulate expression of apoptosis-specific target genes. Given that Myc and Mad have identical *in vitro* DNA-binding specificities, as judged by our selection assays, it is likely that *in vivo* the Myc bHLHZ domain may mediate specific interactions with other proteins, such as Miz-1, that would in turn influence both Myc target gene specificity and transcriptional activity (12).

Conclusions

Our findings support a view of Myc and Mad as transcription factors with identical intrinsic DNA-binding specificities and opposing transcriptional activities. The antagonism between Myc and Mad at the level of proliferation and transformation would be expected to arise from activation or repression at an overlapping set of target genes. Other functions acquired during evolution, such as Miz-1 binding and repression by Myc, would not be subject to antagonism by Mad. Thus, antagonism between Myc and Mad proteins is likely to be partial. Further identification of specific target genes for these transcription factors will be required to delineate precisely their biological functions.

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